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In vitro propagation of endemic and endangered *Muscari mirum* from different explant types

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Abstract: A protocol for in vitro propagation of *Muscari mirum* Speta, an endangered geophyte of the family Hyacinthaceae, was developed. Bulb scale, scape, leaf, and immature embryo explants were cultured on different nutrient media compositions supplemented with various concentrations of plant growth regulators (BAP, NAA, TDZ, Picloram, 2,4-D). Scape explants produced callus only. Higher numbers of bulblets were obtained from bulb scale explants than embryo explants. The highest percentage of the bulb formation (23.50 per explant) were obtained from bulb scale explants consisting of 4 scale segments cultured on Murashige and Skoog (MS) medium supplemented with 4 mg L^{-1} 6-benzylaminopurine (BAP) and 0.25 mg L^{-1} α- naphthaleneacetic acid (NAA) after 5 months of culture initiation. Rooted bulblets which were more than 5 mm in diameter were transplanted into potting mixture of soil, vermiculite and perlite (1:1:1). The chromosome number of the root cells of in vitro induced bulblets was diploid 2n = 18.

Key words: *Muscari mirum*, in vitro propagation, bulb scale, immature embryo

Endemik ve tehlike altındaki *Muscari mirum* türünün farklı eksplant tiplerinden in vitro çoğaltımı

Özet: Bu çalışmada Hyacinthaceae familyasına ait tehlike altındaki geofitlerden *Muscari mirum* Speta türünün in vitro çoğaltılması için bir protokol geliştirilmiştir. *Muscari mirum* türü soğan pul, skap, yaprak ve olgunlaşmamış embriyo eksplantları farklı konsantrasyonlarda bitki büyüme düzenleyicileri (BAP, NAA, TDZ, Picloram, 2,4-D) ile desteklenmiş ve farklı besin ortamlarında kültüre alınmıştır. Skap eksplantından yanlızca kallus oluşumu elde edilmiştir. Soğan pul yaprağı eksplantlarından embriyo eksplantlarına göre daha fazla soğancık elde edilmiştir. En yüksek soğancık oluşumu (eksplant başına 23,50) kültür başlangıcından 5 ay sonra, 4 pul yapraklı soğan eksplantlarından 4 mg L^{-1} 6-benzilaminopurin (BAP) ve 0,25 mg L^{-1} α-naftalenasetik asit (NAA) içeren Murashige Skoog (MS) ortamında elde edilmiştir. Çapı 5 mm'den daha büyük olan köklü soğancıklar 1:1:1 oranında toprak, vermikülit ve perlit karışımına aktarılmıştır. In vitro ortamda üretilen soğanların kök hücrelerinde kromozom sayısının 2n = 18 diploid olduğu tespit edilmiştir.

Anahtar sözcükler: *Muscari mirum*, in vitro üretim, soğan pul yaprağı, olgunlaşmamış embriyo

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Introduction

Turkey is one of the richest countries in variability of flora. It has nearly 9000 plant species about 3000 of which are endemic (Ekim et al., 2000). There are about 600 species of flower bulbs in Anatolia (Arslan et al., 2002) and many of them are known as ornamental and medicinal plants (Atay, 1996). A number of these geophytic species have been exported from Turkey for a long time (Arslan et al., 2002).

The genus *Muscari* Mill., which belongs to the Hyacinthaceae family, has 29 species in Turkey (Eker & Koyuncu 2008; Doğu & Bağcı, 2009). Muscaries are excellent bulbous plants which are generally called grape hyacinths and they deserve a greater breeding effort because of their excellent horticultural characteristic (Nakano et al., 2005). In addition, a number of polyphenolic compounds, which have pharmacological importance because of their antimutagenic effects, have been isolated from some *Muscari* species (Miadokova et al., 2002).

Muscari mirum Speta (mirum means wonderful or extraordinary), which is a perennial ornamental plant, is endemic to Turkey. It grows in organic soil, which is formed from limestone screes and serpentine rocks at 1450-1650 m. It flowers from May to June. The sterile flowers are violet and on short pedicels and with greenish lobes (Özhatay, 2000). This species is represented in the endangered categories in the Red Data Book of Turkish Plant (Ekim et al., 2000) and collection of *M. mirum* from the natural habitats is forbidden in Turkey.

Muscari species can be propagated from seed and bulb division (Mori & Nakano, 2004; Ozel et al., 2007). However, it takes about 3-5 years or longer for a developed plant to be able to flower (Ozel et al., 2007). Since traditional propagation methods are very slow, in vitro micropropagation of this plant will have importance for germplasm conservation and commercial production. Micropropagation of some *Muscari* species such as *M. macrocarpum* Sweet (Ozel et al., 2007), *M. muscarimi* (L.) Medik. (Uranbey et al., 2006), and *M. armeniacum* Leichtlin ex Baker (Suzuki & Nakano, 2001) has been reported. So far no protocol for in vitro propagation of *M. mirum* has been reported.

The present study investigated an efficient bulblet regeneration of *M. mirum* from bulb scale, scape, leaf, and immature embryo explants under in vitro conditions. We also determined the chromosome number of *M. mirum*, which was produced by tissue culture methods.

Materials and methods

Plant material and disinfection

Muscari mirum plants were collected from their natural habitats in Burdur province of Turkey in May. After collection, the leaves and scapes were removed from the bulbs. Leaf and scape explants were washed under running tap water. They were surface sterilised with 95% ethanol for 3 min and 20% commercial bleach for 20 min.

The bulbs were stored in the dark at 5 °C for 6 weeks. After 6 weeks the roots and the outer scales were discarded and the bulbs were washed with detergent thoroughly under running tap water. The scales were dipped in 95% ethanol for 3 min; then they were surface sterilised with 35% commercial bleach for 20 min.

Fruit capsules containing immature embryos were immersed in 80% commercial bleach for 20 min. Then all explants were rinsed 3 times with sterile distilled water.

In vitro culture from leaf and scape explants

After sterilisation, leaves and scapes were cut into about 5 mm length. Explants were cultured on MS medium (Murashige & Skoog, 1962) supplemented with 1.0, 2.0, 4.0 mg L^{-1} BAP (6-benzylaminopurine) and 0.1, 0.5 mg L^{-1} NAA (α-naphthalene acetic acid); 0.5, 1.0, 2.0 mg L^{-1} 2,4-D (2,4-dichlorophenoxy acetic acid) or 0.5, 1.0, 2.0 mg L^{-1} picloram (4-amino-3,5,6-trichloropicolinic acid) and 30 g L^{-1} sucrose and 7 g L^{-1} agar (Sigma type A).

Bulblet regeneration from bulb scale explants

The bulbs of *M. mirum* were longitudinally cut into 4 sections. Bulb scale explants consisting of twin or 4 scale segments attached to a thin segment of the basal plate were isolated. Explants were cultured on MS medium supplemented with 1.0, 2.0, 4.0 mg L^{-1} BAP and 0.25, 0.5 mg L^{-1} NAA; 0.05, 0.1 mg L^{-1} TDZ

(thidiazuron) or 0.05, 0.1 mg L^{-1} TDZ and 0.25 mg L^{-1} NAA and 30 g L^{-1} sucrose and 7 g L^{-1} agar (Sigma type A).

Bulblet regeneration from immature embryo explants

Immature seeds were removed from surface sterilised fruit capsules. Then immature embryos were extracted from immature seeds by squeezing with a pair of forceps. Immature embryos (approximately 1 mm in length) were placed on MS medium supplemented with 1.0, 2.0, 4.0 mg L^{-1} BAP and 0.25, 0.5 mg L^{-1} NAA and 30 g L^{-1} sucrose and 7 g L^{-1} agar (Sigma type A).

The pH of all medium was adjusted to 5.7 before autoclaving at 121 °C for 20 min. The cultures were kept at 25 ± 1 °C and under a 16-h (day)/8-h (night) photoperiod. Explants and explants with bulblets were subcultured every month onto the fresh medium. The bulblets regenerated on bulb scale explants and immature zygotic embryos were separated from the explants and transferred to the MS medium without plant growth regulators for rooting.

Ex vitro culture

Rooted bulblets were washed under running tap water to remove the agar. Then bulblets were transferred into the pots containing sterilised soil, vermiculite, and perlite (1:1:1). Potted plants were maintained in the greenhouse at 24 ± 1 °C under natural light and watered every day for 2 weeks and then twice a week.

Observation and statistical analysis

Each treatment had 3 replicates containing 10 explants for bulb scale and 15 explants for immature embryo. Analysis of variance (ANOVA) and Duncan's multiple range test were performed to analyse the multiplication rates.

Cytological analysis

The root tips of in vitro induced 10 randomly selected bulblets from the MS medium containing 4 mg L^{-1} BAP and 0.25 mg L^{-1} NAA (producing most bulblets) were cut and put into the α-bromonaphthalene for 16 h. Then root tips were put into the glacial acetic acid for 30 min. After that root tips were stored in 70% ethanol in +4 °C. Before staining, root tips were kept in 60% hydrochloric acid for 11 min for hydrolysis and stained with Feulgen for 1 h. A standard Feulgen squash technique was employed (Elçi, 1994; Dirmenci et al., 2010). At least 8-10 good metaphase plates in each plant were counted and photographs of the best somatic chromosome sets were taken.

Results and discussion

In vitro regeneration from leaf and scape explants

In this study we used different types of auxins such as NAA 2,4-D, and picloram and different concentrations of BAP as a cytokinin for bulblet regeneration from leaf and scape explants of *M. mirum*. No bulbs could be achieved from leaf and scape explants. Some 50% of scape explants produced purple calli on MS medium containing 1 or 2 mg L^{-1} 2,4-D, 15 days after culture initiation (Figure 1a). When leaf explants were used as an explant sources, no callus formation were obtained on MS medium supplemented with different kinds and concentrations of plant growth regulators. Mori and Nakano (2004) reported creamy-white callus formation from leaf and flower bud explants of some *Muscari* species on MS medium containing 54 µM NAA. However, they did not obtain any callus formation from the leaf explants of *M. azureum* Fenzl, *M. botryoides* (L.) Mill. 'Album', or *M. paradoxum* K.Koch. In addition, Suzuki and Nakano (2001) analysed the explant types of *M. armeniacum*, and found that the percentage of explants producing calluses was the highest in leaf explants on MS medium containing 4.1 or 41 µM picloram and 4.5 µM 2,4-D. In our study we could not obtain any callus on MS medium supplemented with 1.0, 2.0, 4.0 mg L^{-1} BAP and 0.1, 0.5 mg L^{-1} NAA or 0.5, 1.0, 2.0 mg L^{-1} picloram. Variations in the responses of *Muscari* species might result from the genotype-explant combinations. However, scape explants were more productive than the leaf explants for callus formation; neither of the explant types of *M. mirum* were suitable for bulblet formation in the culture media used in the present study. As a result of their study on *Lilium rubellum* Baker, Niimi and Onozawa (1979) suggest that due to different endogenous hormone balance in leaf of each genotype different growth regulators should be used.

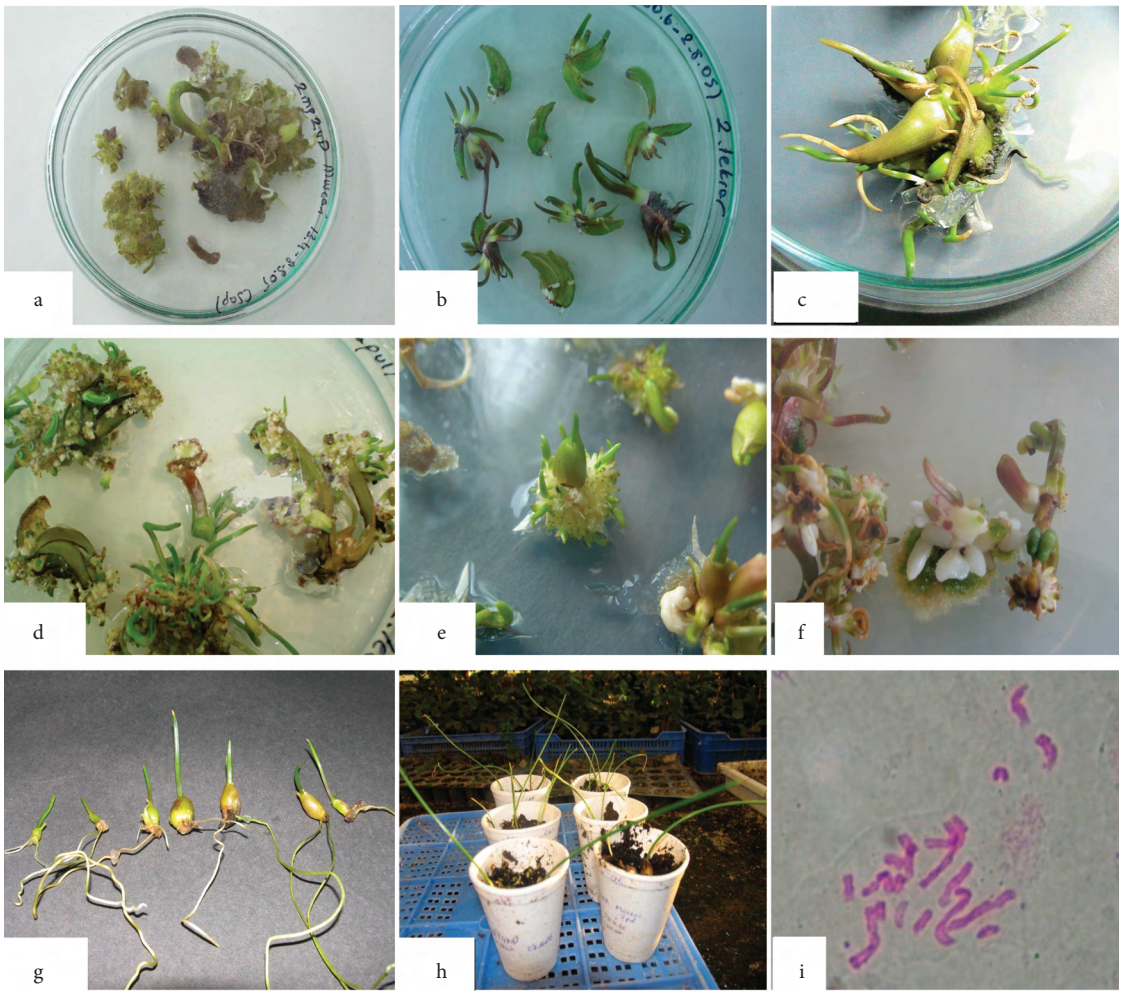

Figure 1. **a**- Callus formation from *Muscari mirum* scape explants on MS medium containing 2 mg L^{-1} 2,4-D; **b**- Bulblet regeneration from 2 scale explants on MS medium containing 0.1 mg L^{-1} TDZ; **c**- Bulblet regeneration from *Muscari mirum* 2 scale explants, on MS medium containing 2 mg L^{-1} BAP and 0.5 mg L^{-1} NAA; **d**- Bulblet regeneration from *Muscari mirum* 2 scale explants, on MS medium containing 4 mg L^{-1} BAP and 0.25 mg L^{-1} NAA; **e**- Bulblet formation from immature zygotic embryo explants on MS medium supplemented with 2 mg L^{-1} BAP and 0.25 mg L^{-1} NAA after 3 months of culture; **f**- Shoot regeneration from immature zygotic embryo explants on MS medium supplemented with 2 mg L^{-1} BAP and 0.5 mg L^{-1} NAA **g**- Rooted bulblets; **h**- Acclimatised bulblets in the greenhouse. **i**- Diploid chromosome 2n = 18.

Bulblet regeneration from bulb scales

Basal medium supplemented with different combinations of NAA, BAP, and TDZ were tested for bulblet regeneration. The bulblets were clearly visible by 2-3 weeks of culture. Bulblets were produced directly on the bulb scales or the basal plates (Figure 1b, c, d). Well developed bulblets from 2 or 4 scale bulb explants were obtained after 5 months in culture. The plant growth regulator type and concentrations influenced the frequency of bulblet formation from both 2 and 4 scale explants (Table 1). The highest bulblet production from 4 scale explants was obtained on MS medium supplemented with 4 mg L^{-1} BAP and 0.25 mg L^{-1} NAA. On this medium 23.50 bulblets per explants were produced. Ozel et al. (2007) reported that the BAP-NAA combination had significant effects on axillary bulblet multiplication of *M. macrocarpum*. They obtained 1.58 to 12.64 bulblets

Table 1. Bulblet regeneration on MS media supplemented with different growth regulators from *Muscari mirum* bulb scale explants consisting 2 and 4 scale explants.

Growth regulators (mg L^{-1})		Mean number of bulblets per explant	
BAP	**NAA**	**2 scales**	**4 scales**
1	0.25	4.17 c [A]	7.67 bc
2	0.25	10.63 b	10.73 abc
4	0.25	4.20 c	23.50 a
1	0.5	6.87 bc	8.83 bc
2	0.5	7.53 bc	10.27 abc
4	0.5	8.27 bc	10.67 abc
TDZ	**NAA**		
0.05	-	7.73 bc	4.10 c
0.1	-	6.43 bc	5.27 c
0.05	0.25	3.37 c	19.13 ab
0.1	0.25	17.00 a	4.73 c

[A] Values within a column followed by different letters are significantly different at the 0.05 level

per explant from 2 scale explants. When 2 scale explants were used, the best result was obtained on MS medium containing 0.1 mg L^{-1} TDZ and 0.25 mg L^{-1} NAA in our study. Besides the studies reporting the cases in which TDZ reduced bulb regeneration (Cha et al., 2002; Mirici et al., 2005), there are also some others reporting TDZ oriented increases (Huetteman & Preece, 1993; McCarton & Staden, 1998). This variation might stem from genotype and/or from the amount of per mole of TDZ (Huetteman & Preece 1993). In the present study high bulb regeneration was not obtained from the media on which TDZ was used as the only growth regulator. However, when TDZ was used with NAA, the number of bulb regeneration per explant was higher (17-19 bulbs). In addition, as is seen in Table 1, the 2 and 4 scale explant types showed higher bulb regeneration on different amounts of TDZ media. The best result in the 2 scale explant types was 0.1 mg L^{-1} TDZ, and it was 0.05 mg L^{-1} TDZ in the 4 scale explant types. This case may result from the variation in the balance of the explant size/endogenous hormone. Similarly, Wright and Alderson (1980) reported higher shoot production in smaller explants.

After 6 months of incubation, bulblets that had formed 2 or 4 scale explants were removed and transferred to the root initiation medium. In spite of the fact that the presence of TDZ has been shown to be inhibitory to rhizogenesis in a number of species (Huetteman & Preece, 1993), in this study after about 3 weeks most bulblets produced roots and were transferred to the soil.

Bulblet regeneration from immature zygotic embryos

Immature embryos were cultured on MS medium supplemented with different combinations of NAA and BAP. Within 10 days of culture immature embryos swelled and after 3 weeks of culture initiation most explants formed morphogenic callus. Explants were subcultured every month onto fresh medium. Small buds were visible on this callus within 5 weeks. These shoots developed into small bulblets (Figure 1e, f). The bulblet regeneration frequency from immature embryos on MS medium supplemented with different combinations of BAP and NAA was variable to the medium (Table 2). There is no significant difference between different growth regulators ($P > 0.05$). However, the highest bulblet regeneration (9.23 bulblets per explants) was obtained on MS medium containing 2 mg L^{-1} NAA and 0.5 mg L^{-1} BAP. Some bulblets rooted in the regeneration medium. Regenerated bulblets were transferred onto hormone-free MS medium for rooting. All the bulblets rooted in this medium and were transferred to the soil (Figure 1g, h).

Table 2. Bulblet regeneration on MS media supplemented with different growth regulators from immature zygotic embryo explants of *Muscari mirum*.*

Growth regulators (mg L^{-1})		Mean number of bulblets per explant
BAP	NAA	
1	0.25	5.46
2	0.25	5.00
4	0.25	7.83
1	0.5	6.97
2	0.5	9.23
4	0.5	9.07

*There is no significant difference between different growth regulators ($P > 0.05$)

Efficient bulblet regeneration has been obtained from immature embryo explants of several other geophytes such as *Stenbergia fischeriana* (Mirici et al., 2005), *Tulipa sintenesii* and *Tulipa karamanica* (Kalyoncu et al., 2006). Uranbey et al. (2006) reported that the immature zygotic embryo explants of *M. muscarimi* produced 12-57 bulblets per explant.

The most important factors affecting in vitro plant regeneration are plant genotype, explants type, and plant growth regulators (Özcan, 2002). In the present study, 5.0 to 9.23 bulblets were produced by the immature zygotic embryo. The number of bulblet per explant was influenced by the explant type and the plant growth regulators tested in this study.

Cytological Analysis

In indirect embryogenesis there is always a risk of somaclonal variations. Moreover, numerical alteration of chromosomes in embryo-regenerated cells shows several abnormalities such as presence of laggards and micronuclei (Mujlip et al., 2005). Chromosome number of *Muscari mirum*, which grows in natural habitats, is 2n = 18 (Özhatay, 2000). In the present study the chromosome number of *M. mirum,* which was produced by tissue culture methods on MS medium containing 4 mg L^{-1} BAP and 0.25 mg L^{-1} NAA, was diploid (2n = 18) (Figure 1i).

There was no numerical alteration of chromosomes such as hyperploids or hypoploids. In previous studies, genetic stability of in vitro produced regenerants has been reported in other ornamental geophytes including *Ruscus hypophyllum* L. (Jha & Sen, 1985), *Ornithogalum umbellatum* L. (Nayak & Sen, 1995), and *Ornithogalum virens* Lindl. (Naik & Nayak, 2005).

It is important for both gene transfer and in vitro flavonoid production to establish an in vitro propagation protocol for *Muscari* species, which have economic potential as an ornamental plant with flavonoid compounds. Moreover, it is also important for the in vitro propagation of endangered and endemic plants such as *M. mirum* for the protection of genetic sources.

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